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Blue Diode and Red He-Ne Lasers Affect the Growth of Anthocyanin Producing Suspension Cells of Apple

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Abstract
Light is an effective factor in cell suspension culture and must be controlled for optimizing cell growth. Growth of anthocyanin producing suspension cells of a red-fleshed genotype of apple (RFA) was assessed in response to blue diode laser (BDL) and red He Ne (RHNL) laser. The suspension cells in L-shaped test tubes were exposed to short-term laser radiation for 20 min in a rotary shaker. The fresh cell weight (FCW), dry cell weight (DCW), cell volume after sedimentation (CVS), cell number (CN) and cell viability (CV) as criteria of cell growth were recorded at 0, 4, 8, 12 days during cell culture. The cell growth was negatively affected in response to BDL and RHNL compared to control and darkness, respectively. The FCW and DCW was enhanced by BDL whereas were not affected by RHNL. Also, only 30.4 $mWcm^{-2}$ intensity of BDL could increase CVS in RFA cells. Changes in CN were not displayed by RHNL and BDL. BDL more than RHNL decreased CV. Cell death rates observed due to BDL and RHNL were 40.42% and 33.67%, respectively. All these results showed that these lasers had diverse effects on FRA cell growth, however, these cells were more sensitive to BDL than RHNL especially in higher intensities, presumably because of its damage to cell membrane leading to cell death.

Keywords: Cell growth, Cell suspension culture, Apple, Red He-Ne laser, Blue diode laser.

Introduction

Recent advances in plant cell culture allow us to establish callus and suspension cultures of useful plants. The differentiated cells in suspension cultures can produce useful secondary metabolites, ranging from natural pigments to pharmaceutical products (Mustafa et al., 2011). Plant cell cultures have been used as a source for several anti-tumor compounds, e.g. vinblastine, vincristine, tripdiolides and podophyllotoxines. Plant cell culture which has a low productivity, can only be economically viable in the production of high-value metabolites. However, concentration of the secondary metabolites in *in vitro* cultures is in the range of 1% to 5% which is considerably lower than that in *in vivo* plants (Park et al., 2003). In order to overcome the low productivity, research in plant cell culture has been dedicated to the selection of cell lines with appropriate genetic biochemical and physiological characteristics, application of elicitation-

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enhancers, and the optimization of growth media. Among the culture conditions, light quality and quantity affect accumulation of useful secondary metabolites. However, one main problem in the exploitation of plant cell culture technique is the lack of knowledge on the effects of light illumination on the production of secondary metabolites (Zhong, 2007). However, there are some reports about the effects of light on the production of flavonoids such as anthocyanins, terpenoids and other metabolites. Generally, in most of these researches the light sources used are fluorescent and LED (Light Emitting Diode) lamps (Stutter et al., 2005). Due to its certain properties such as single wavelength, focus and intensity, laser, which is an abbreviation of "light amplification by stimulation of radiation", can affect physiological and biochemical processes differently in comparison with natural light illumination (Kazemzadeh, 2013). Previously, we had discussed the effects of BDL and RHNL on *in vitro* anthocyanin production in cell suspension culture of a red-fleshed apple (RFA) and indicated that both BDL and RHNL significantly could enhance anthocyanin production, having the BDL more effective than RHNL (Kazemzadeh, et al., 2015; Kazemzadeh et al., 2017). Nevertheless, the effects of these lasers on the growth of RFA suspension cells is yet to be assessed which is the main focus of the current research.

Materials and methods

Plant material and callus induction

Plant material was prepared from a mature red-fleshed apple tree which is postulated to be a hybrid of *Malus pumila* var. Niedzwetzkyana (Van Nocker et al., 2012) located in Shahid Bakeri Agriculture Educational Center of West Azerbaijan province of Iran. Buds of this tree were decontaminated using the procedure previously described by Dobranszki et al. (2010). Shoot tips including some leaf primordia were then cultured in the MS medium supplemented with 1.0 mg/l benzyl adenine (BA), 100 mg/l FeEDDHA, 3 g/l Polyvinylpyrrolidone (PVP) and 3% sucrose (w/v). After sufficient growth of buds, the seedlings were proliferated in the same culture medium and their leaves were used as explants for callus production (Mahna and Motallebi-Azar, 2007). The medium for callus induction was a modified MS supplemented with 3% sucrose (w/v), 100 mg/l *myo*-inositol, 0.5 mg/l kinetin, 1.5 mg/l 2,4-D and 1 mg/l NAA (Naphthalenecetic acid). All cultures were maintained in dark condition at 23±2 °C. It was sub-cultured every four weeks on the modified MS medium.

Plant cell culture

Cell suspension cultures were started by putting 2 g of fresh friable callus tissue in 100 ml liquid MS medium supplemented with 3% (w/v) sucrose, 1.2 mM asparagine, 284 µM ascorbic acid, 328 µM thiourea, 555 µM *myo*-inositol, 2 µM Ca pantothenate, 8 µM nicotinic acid, 3 µM thiamin, 4.8 µM pyridoxin HCL, 0.04 µM biotin and 1 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D) in a 250 ml flask (Codron et al., 1979). They were incubated on a rotary shaker at 80 rpm under continuous fluorescent light of 24 µmol m^{-2} s^{-1} at 23±2 °C for 3 weeks, during which the medium was changed every week (Kurata et al., 2000). Afterwards, this suspended callus tissue was filtered through a mesh with a pore size of 53 µm and obtained cell suspension was transferred into L-shaped test tubes with 2 cm diameter under the same light condition on a shaker and after 15 days exposed to the defined lasers for 20 min.

Blue diode laser (BDL) and red He Ne (RHNL) lasers radiation treatments

The lasers were constructed at Faculty of Physics, University of Tabriz (a RHNL: wavelength: 630-640 nm, power intensity: 5 mW, radiation diameter: 0.5 x 1.5 mm^2 and a BDL: wavelength: 437 nm, power intensity: 50 mW, radiation diameter: 0.5 x 1.5 mm^2).

This experiment was carried out with ten radiation treatments including exposing the cells to BDL at the intensities of 67.09, 32.74, 30.4 and 13.73 mW cm^{-2} (designated as BDL-I1, BDL-I2, BDL-I3 and BDL-I4, respectively), and to RHNL at the intensities of 6.46, 4.82, 1.54 and 0.66 mW cm^{-2} (shown as RHNL-I1, RHNL-I2, RHNL-I3 and RHNL-I4, respectively) as well as two controls including darkness control (no exposure) and fluorescent light control with an intensity of 24 µmol m^{-2} s^{-1} with three replications for each treatment in a completely randomized design (CRD). At first, after evaluation of cell suspension cultures exposed to BDL and RHNL for *in vitro* production of anthocyanin (Kazemzadeh at al., 2015), these cultures were investigated for their growth characteristics mentioned below. Data were subjected to analysis of variance (ANOVA) and calculations and statistical analyses were carried out using SAS (Statistical Analysis System, SAS Institute Inc., 1985) Portable ver.9.1.3 software. Mean comparisons were performed using the Tukey's or HSD (Honestly Significant Difference) test.

Measurement of cell growth

The samples were taken from cell suspensions cultured in L-shaped test tubes in two replications (one ml per each replication) 0, 4, 8 and 12 days after treatment with lasers. The samples were centrifuged at 3,000 rpm for 15 min. The precipitated cells were then washed with distilled water, centrifuged again at 10,000 rpm for 15 min and weighed using an electronic weighing balance with an accuracy of ± 0.01 g to get the fresh cell weight (FCW). The cells were then dried at 60 ºC for 24 h in a ventilating oven to determine dry cell weight (DCW) (Mustafa et al., 2011).

For cell volume after sedimentation (CVS) assay, the cells were settled for 20 min. Then the fraction of the whole culture volume occupied by the cell mass was measured using a simple gradation ruler and determined as the CVS (Mustafa et al., 2011).

To distinguish between the living and dead cells after laser treatment, cell cultures were stained with a standard trypan blue dye exclusion. The cell number (CN) and cell viability (CV) were measured by a light microscope on a hemocytometer slide (Improved Neubauer Hemocytometer Blood Cell Counting Chamber, Jack Electronic Company, China) with an identical chamber of precise dimensions. Trypan blue solution 0.4% was prepared in 0.81% sodium chloride and 0.06% dibasic potassium phosphate. One ml of 2-week-old cells was centrifuged at 12000 rpm for 10 min. The cell debris was then diluted with distilled water to prepare a mixture containing the 30 µl of suspension cells and the 30 µl of 0.4% Trypan blue solution (1:1v/v). The mixture gently mixed and let stand for 5 min at room temperature. The 15 µl of mixture applied to the edge of the chamber between the cover slip and the V-shaped groove in the chamber and then the suspension cells allowed to be drawn into the chamber by capillary action. Finally, the manually counting of CN and CV (living cells: unstained; dead cell: stained) were determined according to the method of Hou and Lin (1996).

Results

Assessment of cell growth and viability is important in cell suspension cultures to guarantee the reproducibility of production in any scale from a small lab to large-scale industrial productions. Nevertheless, there is no unique protocol for precise measurement of cell growth in plant cell suspension cultures, but there are some parameters that have been measured in most of studies including FCW, DCW, CN, CV, CVS, packed cell volume, loss of weight by dissimilation, DNA content, protein content, and so forth (Mustafa et al., 2011). In the present study, we have measured the FCW, DCW, CVS, CN and CVS as cell growth parameters. On the other hand, laser radiation may exert

significant effects through stimulating stress responses and influencing biological processes in the exposed cells, here, the RFA cells. Results showed that the BDL and RHNL had a significant effect on *in vitro* growth of RFA cells (Table 1). The results for FCW, DCW, CVS, CN and CV parameters have been illustrated in Table 2. The suspension cells exposed to RHNL did not display a significant increase in FCW and DCW even at the highest intensity as compared to darkness and light controls. In contrast, these parameters were increased by BDL especially the BDL-I2. Overall, the BDL came up to be more effective for FCW and DCW through cell suspension culture than RHNL. A little increase in the intensity of BDL up to I2 could increase these parameters, however, with increasing the intensity of BDL, FCW and DCW exhibited a decreasing trend, which is dependent on intensity to BDL.

The mean comparison with Tukey's test indicated the RHNL had a significant difference in the CVS with darkness control but not with light control. However, exposing the RFA cells to BDL resulted in a higher CVS. The highest CVS was obtained from BDL-I2 while the lowest CVS occurred in darkness and BDL-I1, respectively. It can also be noticed that all of the intensities of RHNL had the same effects on CVS and has no significant difference with control as well (Table 2).

Table 1. Analysis of variance for determining the plant cell growth of red fleshed-apple cell suspension culture exposed to red He-Ne laser (RHNL) and blue diode-laser (BDL).

Sources of variation	df	MS				
		CN	CV	CVS	FCW	DCW
Lasers radiation	9	266.85**	234.47*	0.794*	180.35 $^{n.s}$	218.62**
Duration cell growth	3	100.24*	126.67**	1.71**	284.79*	980.81**
Lasers radiation × Duration cell growth	27	34.8 $^{n.s}$	48.91*	0.16**	202.40*	83.15**
Error experiment	27	39.84	45.91	0.011	96.89	23.02

Note. The ns, *, ** represent non-significant and significant at 5% and 1% levels respectively.

Table 2. Mean comparisons of effect of red He-Ne-laser (RHNL) and blue diode-laser (BDL) on cell growth parameters including FCW, DCW, CVS, CN and CV from red fleshed apple cell suspension.

Lasers treatments	Plant Cellular Growth Parameters				
	CN ($\times 10^4$)	CV (%)	CVS (cm)	FCW (mg/ml)	DCW (mg/ml)
Darkness	47.70 ± 12.33 c	69.50 ± 4.00 ab	1.46 ± 0.19 d	21.97 ± 5.94 c	18.42 ± 9.75 b
Control	52.16 ± 5.53 abc	70.25 ± 2.08 ab	2.00 ± 0.31 ab	28.71 ± 13.16 bc	21.48 ± 3.95 b
RHNL-I1	58.87 ± 4.50 a	68.25 ± 5.68 abc	1.93 ± 0.10 bc	27.76 ± 1.94 bc	23.62 ± 1.33 b
RHNL-I2	52.37 ± 1.15 abc	72.08 ± 1.73 a	1.87 ± 0.09 bc	26.70 ± 3.79 bc	20.84 ± 1.28 b
RHNL-I3	53.37 ± 1.04 abc	67.91 ± 7.21 bc	1.94 ± 0.18 bc	26.53 ± 2.27 bc	23.43 ± 0.98 b
RHNL-I4	50.66 ± 5.50 bc	66.33 ± 1.73 bc	1.96 ± 0.18 bc	26.05 ± 3.44 bc	19.39 ± 4.28b
BDL-I1	49.37 ± 2.64 bc	59.58 ± 5.77 ef	1.53 ± 0.28 cd	23.15 ± 6.33 c	19.28 ± 6.57 b
BDL-I2	55.87 ± 7.97 ab	65.08 ± 1.00 cd	2.36 ± 0.76 a	38.90 ± 14.40 a	33.78 ± 14.24 a
BDL-I3	46.54 ± 2.78 c	61.91 ± 4.93 de	1.71 ± 0.21 bcd	31.50 ± 11.04 ab	25.10 ± 8.83 b
BDL-I4	47.91 ± 11.35 bc	60.58 ± 8.38 ef	1.97 ± 0.18 ab	28.95 ± 1.61 bc	23.60 ± 1.07 b

Mean ± SE, $n = 30$. Mean values with different letters in the same column are significantly different at $P < 0.05$. FCW: fresh cell weight, DCW: dry cell weight, CVS: cell volume after sedimentation, CN: cell number, CV: cell viability. Darkness (control): no radiation/light, Control (light control): fluorescence light illumination with an intensity of 24 µmol m^{-2} s^{-1}. I1 to I4 represent four intensity levels for each laser.

Although RHNL-I1 and BDL-I2 had a significant difference in the CN of the cells with darkness control, this significant affect cannot be explained by laser radiation, because they had no difference with light control. The highest CN level was observed in RHNL-I1 but lowest CN level obtained from BDL-I3. In other words, both the RHNL and the BDL were unable to enhance the CN level of RFA cells more than light control.

The results showed that both the RHNL and the BDL led to a decrease in the CV% (Table 2). A reverse relationship was observed between CV% with RHNL and BDL. It has been assumed that laser radiation can directly influence cell processes such as transferability of water, ions, and metabolites through changing permeability of cell membranes (Anisimov et al., 1997). This was attested by the relationship between the intensity of the BDL and CV%. Regarding RHNL, only two intensities (RHNL I3, RHNL I4) had led to decrease in the CV% wherever other levels (RHNL I1, RHNL I2) were similar to light and darkness controls. It should be noticed that BDL was more effective than RHNL in decreasing the CV% in RFA cells. Despite that the BDL (BDL-I2) had a significant effect on the CN%, it could decrease CV% in comparison to the controls and exert damages and even death to the RFA suspension cells as it is obvious in Figure 1. The death of RFA suspension cells was probably associated with changes in the plant cellular structure and metabolism, e.g. dilation of thylakoid membranes, modulation of the anti-oxidative system, and accumulation of phenolic compounds, which these results are in agreement with finding of Chung et al. (2005). Also, accumulation of anthocyanin, as a phenolic compound, induced by high intensity of BDL in RFA cell in our previously work is confirmed with these results (Kazemzadeh et al., 2015).

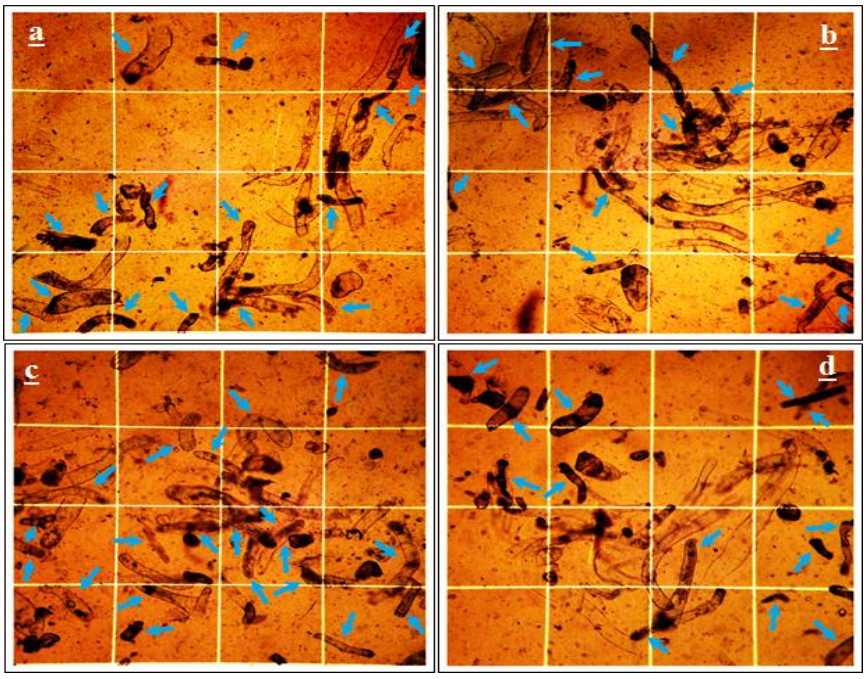

Fig. 1. Effect of the red He-Ne-laser (RHNL) and blue diode-laser (BDL) radiation on the cell number and cell viability from red-fleshed apple cell suspension culture. Here, negative effects of higher intensities of RHNL (left) and BDL (right) on cell viability have been shown. The phrases a, b, c and d represent control (fluorescence light illumination), RHNL I4 (6.46 mW cm^{-2}), BDL I3 (32.74 mW cm^{-2}) and BDL I4 (67.09 mW cm^{-2}), respectively. The dark colored cells represent dead cells (stained by trypan blue: blue arrow), while the viable cells are white (unstained). It is necessary to notice that the semi-dark cells were considered as dead cells.

In this experiment, the interaction between laser radiations and cell growth was significant for FCW ($p< 0.05$), DCW ($p< 0.01$), CVS ($p< 0.01$), and CV % ($p< 0.01$) parameters, whereas it did not significantly influence the CN (Table 1). As shown in Figure 2A, the RHNL could not enhance the FCW in RFA cells compared to the controls at end culture. The FCW was only increased in response to BDL after 12 days. Best result for DCW was obtained after 4 days with BDL-I2; then a sharp reduction occurred at 8th day and increased again at 12th day (Figure 2B). The results demonstrated that, the CVS had an increase for control and RHNL treatments at 12th day, while for BDL the increase was not noteworthy (Figure 2C). On the last sampling data, the CVS of RHNLs were significantly higher than BDLs. At the 4th day, the highest and lowest levels of CN was attributed to RHNL I1 and darkness, respectively. The I1 intensity of RHNL could increase the CN, but with increasing the intensity of this laser, CN had a decreasing trend. However, for BDL, the changes in CN was not so worthy of attention. This trend for both lasers were similar in all days when CN was measured (Figure 3A). As expected, the CV% was greatly decreased by both the BDL and the RHNL compared to the control at 12th day. Decreasing in CV% by BDL was higher than that by RHNL (Figure 3B).

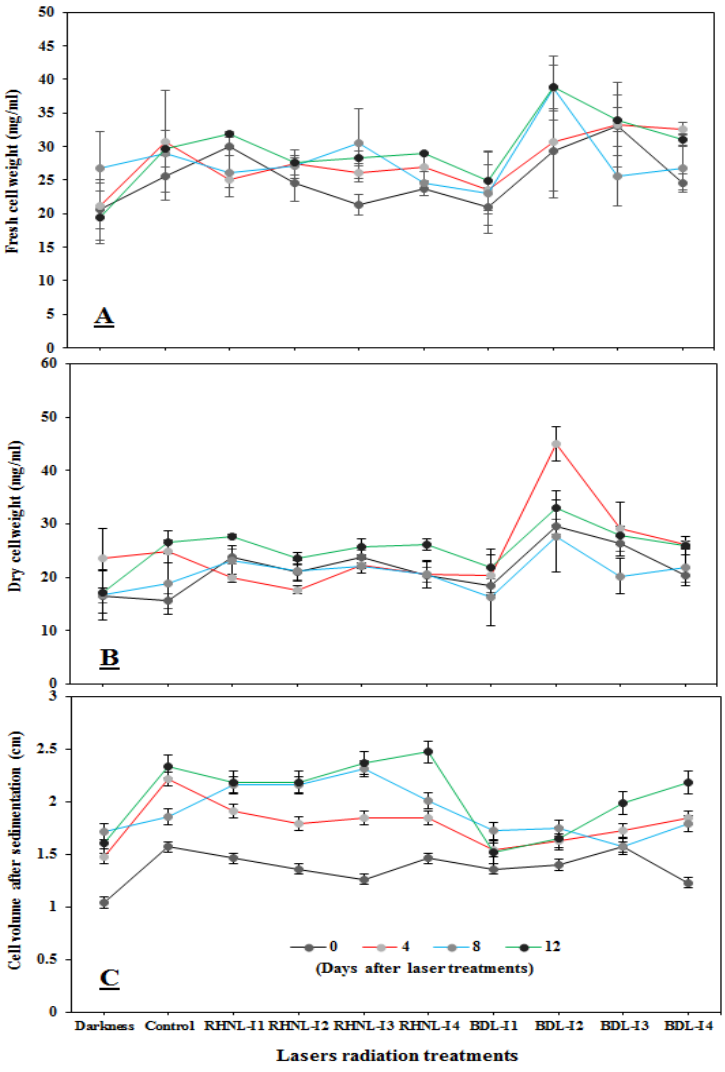

Fig. 2. Evaluation of the effects of red laser-He-Ne (RHNL) and blue laser-diode (BDL) on cell growth parameters. Data are means of three replications. Bars indicate standard deviations.

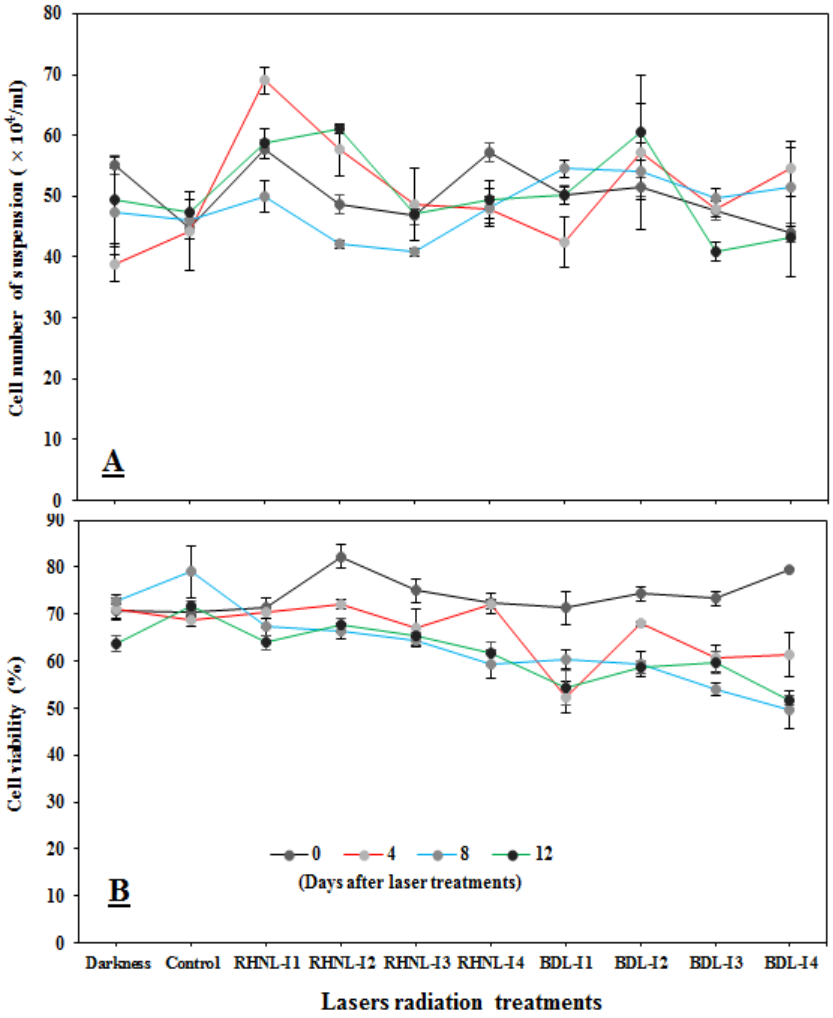

Fig. 3. Evaluation of the effects of red He-Ne-laser (RHNL) and blue diode-laser (BDL) on cell number and cell viability of red-fleshed apple cell suspension culture. Data are means of three replications. Bars indicate standard deviations.

Discussion

Based on the investigations performed so far, we believe that there is no noteworthy study on the evaluation of laser radiation on plant cell growth in suspension cultures. It has been suggested that when a cell receives laser light, it shows two specific responses: a rapid stress effect increasing lipid peroxidation, and long-lasting reactions leading to some metabolic changes and apparently the stimulation of morphogenetic processes (Salyaev et al., 2007).

There is no clear explanation concerning the effect of laser radiation on biological materials due to difficulties in the analysis of light-energy transformation in cells and integrated response of complex multilevel living systems to laser radiation (Ruvinov, 2003). The results here demonstrated clearly that BDL, due to its high energy (high dosage) and low wavelength, could induced diverse effects on the growth of RFA suspension cells.

Plants respond to laser irradiation through tolerating some changes in their morphological, physiological, and biochemical characteristics as well as molecular changes. Most of studies have previously highlighted the great complexity of plant response to laser irradiation, as it can affect both the cell and the organism to such varying degrees (Perveen et al., 2010). However, it has been well-known that the effect of laser

radiation on biostimulation can be positive, negative, or none (Ruvinov, 2003). In this respect, we found that the high intensity of BDL displayed a positive effect on FCW, DCW and CVS, while showing no or a negative effect on CN and a negative influence on CV. For the low intensity laser, RHNL, no effect was seen on FCW and DCW, a positive influence witnessed on CVS, and finally no or a positive impact on CN and no or a negative effect on CV.

The laser radiation based on its wavelength, intensity and spatial and temporal coherence, exerts an anisotropic effect on cell and induces directed changes in cell structure (Ruvinov, 2003). As laser irradiation interacts with atoms or molecules, depending on its intensity it can produce free radicals in the affected cells and these radicals can damage or change the plant cellular structure and metabolism; e.g. dilation of thylakoid membranes, alteration in photosynthesis, modulation of the anti-oxidative system, and accumulation of phenolic compounds in plants (Wi et al., 2005). Our previous works on *in vitro* anthocyanin production from RFA suspension cells have proved this concept (Kazemzadeh et al., 2015; Kazemzadeh et al., 2017). Since the intensity of BDL applied in the present study was about 10 times more than RHNL, it would make sense to see the former more effective than the latter. In this way, the results showed that increasing the intensity of BDL from 13.73 to 67.09 mW cm^{-2}, led to a decrease in the CVS, CN and CV% and ultimately caused cell death. The experiments with *Candida albicans* exposed to a laser light (685 nm) intensity of 28 J cm^{-2} also induced 88.6% cell death and a laser light (660 nm) of 2.04 J cm^{-2} induced 42% cell death (Wilson and Mia, 1993). While, in this report, BDL and RHNL radiation induced 40.42% and 33.67% cell death in RFA suspension cells, which is a little milder.

In general, from the results of the present study, it can be suggested that BDL (437 nm) and RHNL (630-640 nm) radiations could prevent the normal growth of RFA suspension cells. These results are in concordance with the photodynamic effect of red light-emitting diode light on cell growth inhibition that can effectively inhibit bacteria, yeast and microcrustacean growth (Peloi et al. 2008). Accordingly, Kurata et al. (2000) stated that both blue and red light were not able to improve cell growth in strawberry cell suspension cultures.

Our study can be considered as the first one in its type on investigating *in vitro* growth behavior of red-fleshed apple suspension cells under the influence of BDL and RHNL radiations and paves the way for the future researches seeking plant cell reactions to laser lights. However, further researches yet to be done to clarify the mechanism through which laser radiation affects these cells.

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